

FORM PTO-1390 (REV 11-98)	U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE	ATTORNEY'S DOCKET NUMBER 1303-102
TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371		U.S. APPLICATION NO. (If known, see 37 C.F.R. 1.5) 09/601513 Unknown
INTERNATIONAL APPLICATION NO. PCT/EP99/00618	INTERNATIONAL FILING DATE 1 February 1999	PRIORITY DATE CLAIMED 3 February 1998
TITLE OF INVENTION METHOD FOR THE DETERMINATION OF PROSTHETIC INFECTIONS		
APPLICANT(S) FOR DO/EO/US THALLER et al		
Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:		
1. <input checked="" type="checkbox"/> This is a FIRST submission of items concerning a filing under 35 U.S.C. 371.		
2. <input type="checkbox"/> This is a SECOND or SUBSEQUENT submission of items concerning a filing under 35 U.S.C. 371.		
3. <input checked="" type="checkbox"/> This is an express request to begin national examination procedures (35 U.S.C. 371(f) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(1).		
4. <input checked="" type="checkbox"/> A proper Demand for International Preliminary Examination was made by the 19 th month from the earliest claimed priority date.		
5. A copy of the International Application as filed (35 U.S.C. 371(c)(2)).		
a. <input type="checkbox"/> is transmitted herewith (required only if not transmitted by the International Bureau).		
b. <input checked="" type="checkbox"/> has been transmitted by the International Bureau.		
c. <input type="checkbox"/> is not required, as the application was filed in the United States Receiving Office (RO/US).		
6. <input type="checkbox"/> A translation of the International Application into English (35 U.S.C. 371(c)(2)).		
<input type="checkbox"/> Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3)).		
a. <input type="checkbox"/> are transmitted herewith (required only if not transmitted by the International Bureau).		
b. <input type="checkbox"/> have been transmitted by the International Bureau.		
c. <input type="checkbox"/> have not been made; however, the time limit for making such amendments has NOT expired.		
d. <input type="checkbox"/> have not been made and will not be made.		
8. <input type="checkbox"/> A translation of the amendments to the claims under PCT Article 19 (U.S.C. 371(c)(3)).		
9. <input type="checkbox"/> An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).		
10. <input type="checkbox"/> A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).		
Items 11. To 16. Below concern document(s) or information included:		
11. <input type="checkbox"/> An Information Disclosure Statement under 37 C.F.R. 1.97 and 1.98.		
12. <input type="checkbox"/> An assignment document for recording. A separate cover sheet in compliance with 37 C.F.R. 3.28 and 3.31 is included.		
13. <input checked="" type="checkbox"/> A FIRST preliminary amendment.		
<input type="checkbox"/> A SECOND or SUBSEQUENT preliminary amendment.		
14. <input type="checkbox"/> A substitute specification.		
15. <input type="checkbox"/> A change of power of attorney and/or address letter.		
16. <input checked="" type="checkbox"/> Other items or information. PTO-1449 and International Search Report		

U.S. APPLICATION NO. (If known, see 37 C.F.R. 1.5) 097/601513 Unknown		INTERNATIONAL APPLICATION NO PCT/EP99/00618		ATTORNEY'S DOCKET NUMBER 1303-102	
17. <input checked="" type="checkbox"/> The following fees are submitted:				CALCULATIONS PTO USE ONLY	
BASIC NATIONAL FEE (37 C.F.R. 1.492(a)(1)-(5)): -- Neither international preliminary examination fee (37 C.F.R. 1.482) nor international search fee (37 C.F.R. 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO\$970.00 -- International preliminary examination fee (37 C.F.R. 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO.....\$840.00 -- International preliminary examination fee (37 C.F.R. 1.482) not paid to USPTO but international search fee (37 C.F.R. 1.445(a)(2)) paid to USPTO\$690.00 -- International preliminary examination fee paid to USPTO (37 C.F.R. 1.482) but all claims did not satisfy provisions of PCT Article 33(1)-(4).....\$670.00 -- International preliminary examination fee paid to USPTO (37 C.F.R. 1.482) and all claims satisfied provisions of PCT Article 33(1)-(4).....\$96.00 <div style="text-align: right;">ENTER APPROPRIATE BASIC FEE AMOUNT =</div>				<div style="border: 1px solid black; padding: 2px;">\$ 840.00</div>	
Surcharge of \$130.00 for furnishing the oath or declaration later than <input type="checkbox"/> 20 <input checked="" type="checkbox"/> 30 months from the earliest claimed priority date (37 C.F.R. 1.492(e)).				<div style="border: 1px solid black; padding: 2px;">\$ 130.00</div>	
CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE		
Total Claims	14	-20 = 0	X \$18.00	\$ 0.00	
Independent Claims	4	-3 = 1	X \$78.00	78.00	
MULTIPLE DEPENDENT CLAIMS(S) (if applicable)			\$260.00	\$ 0.00	
TOTAL OF ABOVE CALCULATIONS =				\$ 1048.00	
Reduction by 1/2 for filing by small entity, if applicable. A Small Entity Statement must also be filed (Note 37 C.F.R. 1.9, 1.27, 1.28).				0.00	
SUBTOTAL =				\$ 1048.00	
Processing fee of \$130.00, for furnishing the English Translation later than <input type="checkbox"/> 20 <input type="checkbox"/> 30 months from the earliest claimed priority date (37 C.F.R. 1.492(f)).				0.00	
TOTAL NATIONAL FEE =				\$ 1048.00	
Fee for recording the enclosed assignment (37 C.F.R. 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 C.F.R. 3.28, 3.31). \$40.00 per property				0.00	
Fee for Petition to Revive Unintentionally Abandoned Application (\$1210.00 - Small Entity = \$605.00)				0.00	
TOTAL FEES ENCLOSED =				\$ 1048.00	
				Amount to be:	
				refunded \$	
				Charged \$	
a. <input checked="" type="checkbox"/> A check in the amount of \$1048.00 to cover the above fees is enclosed. b. <input type="checkbox"/> Please charge my Deposit Account No. 14-1140 in the amount of \$_____ to cover the above fees. A duplicate copy of this form is enclosed. c. <input checked="" type="checkbox"/> The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 14-1140. A duplicate copy of this form is enclosed. d. <input checked="" type="checkbox"/> The entire content of the foreign application(s), referred to in this application is/are hereby incorporated by reference in this application. NOTE: Where an appropriate time limit under 37 C.F.R. 1.494 or 1.495 has not been met, a petition to revive (37 C.F.R. 1.137(a) or (b)) must be filed and granted to restore the application to pending status.					
SEND ALL CORRESPONDENCE TO: NIXON & VANDERHYE P.C. 1100 North Glebe Road, 8 th Floor Arlington, Virginia 22201 Telephone: (703) 816-4000					
<div style="border-bottom: 1px solid black; display: inline-block; width: 200px;"> </div> SIGNATURE					
Arthur R. Crawford NAME					
25,327 REGISTRATION NUMBER					
August 2, 2000 Date					

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of

THALLER et al

Atty. Ref.: 1303-102

Serial No. **Unknown**

Group:

National Phase of **PCT/EP99/00618**Filed: **August 2, 2000**

Examiner:

For: **METHOD FOR THE DETERMINATION OF PROSTHETIC
INFECTIONS**

* * * * *

August 2, 2000Assistant Commissioner for Patents
Washington, DC 20231

Sir:

PRELIMINARY AMENDMENT

Prior to calculation of the filing fee and in order to place the above identified application in better condition for examination, please amend the claims as follows:

IN THE CLAIMS

Claim 3, line 1, delete "claims 1-2" and insert -- claim 1 --.

Claim 6, line 1, delete "claims 1-5" and insert -- claim 1 --.

Claim 7, line 1, delete "claims 1-6" and insert -- claim 1 --.

Claim 10, line 1, delete "claims 1-5" and insert -- claim 1 --.

REMARKS

The above amendments are made to place the claims in a more traditional format.

Respectfully submitted,

NIXON & VANDERHYE P.C.By: **Arthur R. Crawford**

Reg. No. 25,327

ARC:Imy

1100 North Glebe Road, 8th Floor
Arlington, VA 22201-4714
Telephone: (703) 816-4000
Facsimile: (703) 816-4100

09/601513

METHOD FOR THE DETERMINATION OF PROSTHETIC
INFECTIONS

The present invention refers to a method for the determination of prosthetic infections in which at least one *Staphylococcus* strain is involved. The method is based on the detection of antibodies reacting with a slime polysaccharide produced by virulent staphylococcal strains, from blood samples or other biological fluid samples.

The invention also provides a process for preparing a polysaccharide to use in the above method, starting from cultures of virulent staphylococcal strains, as well as the polysaccharide itself which is obtained by the cited process.

Prosthetic infections represent a severe problem in general surgery, cardiovascular surgery, orthopedics, ophtalmology and odontology, all sectors in which the introduction of biomaterials has become routinary. Prosthetic devices are also widely used in oncology for artificial nutrition and chemotherapy.

The most significant aspects of prosthetic infections are the following:

- their incidence is generally reported to be 2 to 6% of the cases, following the first introduction of the biomaterial;
- when a biomaterial is substituted by a new one, as a consequence of an infection, the incidence of re-infections is about 60%;
- infections may cause functional loss of organs or their parts or even death of the patient in 25 to 45% of the cases, although in some particular cases, as for cardiac surgery, mortality is much higher and in some fields of medicine, as for example odontology, no mortality is usually associated with prosthetic

infections;

- hospitalization of patients is frequently very long and expensive;
- diagnosis of these infections is difficult for the almost
- 5 complete absence of specific clinical signs;
- the greatest part of these infections (over 85%) is caused by coagulase negative staphylococci (and to a lesser extent by coagulase positive staphylococci), although other microorganisms, as enteric bacteria, pseudomonads and enterococci, can also be isolated in various cases (frequently in
- 10 association with at least one staphylococcal strain).

While causing these infections bacteria are strongly glued to the surface of biomaterials, forming colonies that evolve with time in biofilms that may almost entirely cover the surface of the biomaterial, biasing its

15 tissue integration.

Following adhesion bacteria undergo important metabolic modifications, reducing their replicative rate, though maintaining a high biosynthetic activity. The main products of this activity are extracellular polysaccharides (generally indicated as slime) that form a thick and dense

20 fibrous layer protecting bacterial cells from chemicals, radiations, phagocytes, and antibodies.

Slime-embedded bacteria, growing on biomaterials, when exposed to antibiotics, exhibit minimal inhibitory and minimal bactericidal concentrations that can be even hundreds of times higher than the

25 corresponding values obtained when bacteria are grown in the absence of the biomaterial.

When a biofilm reaches a determined critical mass, it releases in the surrounding liquid environment little aggregates of bacterial cells that may

move to colonize new surfaces of the biomaterial.

Prosthetic infections are frequently characterized by scarce and non specific clinical signs, that are most often confused with minor viral episodes, and that disappear following common antibiotic therapy to
5 manifest again after some time. These episodes are generally due to the release of small bacterial aggregates from the biofilm, that while circulating in blood are easily recognized by the immune defenses of the host, causing fever, and that, being in the planktonic form, are easily killed by common antibiotics, thus mimicking the eradication of the infection. Clinical signs
10 become evident in the advanced phases of the infection, with formation of large tumefactions of the soft tissues surrounding the graft, or fistulas, or evident signs of general compromission.

Due to this peculiar characteristics early diagnosis is extremely difficult, even as a consequence of the high incidence of false negatives
15 following cultural microbiological analyses, depending on the fact that sessile bacteria are not able to rapidly adapt to in vitro cultural conditions.

Till now diagnosis of vascular graft infections has been based on the analysis of objective clinical parameters, blood parameters, and instrumental data obtained by ultrasonography, computed tomography,
20 magnetic resonance, esophago-gastro-duodenoscopy, and scintigraphy following injection with radio-labelled leukocytes. This way it is in most cases not possible to reveal the early phases of infection for the insufficient sensitivity or for the lack of well defined criteria of interpretation of data and images.

25 Overall the actual diagnostic possibilities are absolutely inadequate, particularly in the early phases of the disease that are more favourable for a successful therapy.

It has now been found, and this is the object of the present invention,

a reliable and unexpensive method, that can be easily applied to serum samples or other biological fluids, able to reveal the presence of infections involving prosthetic devices even in the early phases of development. This method can be routinely performed for the constant monitoring of patients
5 that were treated with the introduction of any kind of prosthetic device and are at risk for infection, especially caused by staphylococci.

This method consists in the quantitative determination of the presence of antibodies, specifically directed against extracellular polysaccharides, extracted from virulent strains of staphylococci.

10 Furthermore, the present invention provides a method for preparing and purifying these extracellular polysaccharides from adequately grown staphylococcal cells.

Staphylococcal strains that may be used for this method are both coagulase-negative and positive, either virulent ones or anyway able to
15 produce slime. In particular, virulent *Staphylococcus epidermidis* or *Staphylococcus aureus* strains are preferred.

These strains can be obtained either directly from patients suffering from a prosthetic infection or from standard culture collections of reference strains. The characteristics of such a typical strain are reported in the
20 following Example 1. Experiments were also indicative when performed using the *Staphylococcus aureus* strain deposited by the applicant at DSMZ No. 11942.

Bacteria are grown in the liquid medium described in Hussain et al., J.Med.Microbiol. 34:143-147, 1991 (HHW medium), with some
25 modifications as reported in the following Example 2.

The procedure to prepare the polysaccharides according to the invention essentially involves the following steps:

a) culturing the staphylococcal strains in the modified HHW

medium for a period of 4-6 days;

- b) homogenizing the bacterial cells in a physiological buffer
- c) centrifugating at 13,000 x g for 15 minutes and separating the supernatant;
- 5 d) desalting by dialysis the supernatant using membranes with a cut-off of 12 kDa;
- e) freezing and lyophilizing the solution obtained in (d);
- f) suspending the lyophilized material in a deproteinizing solution, for example trichloroacetic acid;
- 10 g) centrifugating at 30,000 x g the solution obtained (f) and separating the supernatant with addition of ethanol;
- h) centrifugating the supernatant of step (g) at 20,000 x g to obtain the polysaccharide;
- i) washing the precipitated polysaccharide with absolute ethanol,
- 15 dehydrating in vacuo and suspending it in sterile H₂O.

Quantification of the purified polysaccharide could for example be performed according to the method described by Pelkonen et al., Journal of Bacteriology 170, 2646, 1988.

The polysaccharide obtained according to the above described method is used in the assay for the determination of antibodies in serum or other biological fluids from patients having a prosthetic device inserted.

Preferably the assay determines the presence of antibodies of the class IgG and/or IgM, following conventional immunochemical procedures, as for example enzyme linked immunosorbent assay (ELISA), gel immuno-precipitation, immuno-diffusion or counter-immuno-electrophoresis, radioimmunoassay, complement fixation, and passive haemoagglutination.

A solid phase method is preferred in which the polysaccharide is immobilized to a solid surface, as for example microtiter wells and then

allowed to react with the sample, in conditions appropriate for the formation of the immune complex. Once the immune complex has formed it can be revealed by reaction with appropriate molecules, as for example antibodies or their immunocompetent fragments, labelled with radioactive isotopes, chemiluminescent or fluorogenic substances, or coupled to enzymes catalyzing colorimetric reactions and other similar substances.

The efficiency of the method disclosed by the invention was confirmed by the following experiments.

In a first set of experiments sera from 15 patients with a vascular graft infection were analyzed. The presence of a prosthetic infection in these patients was confirmed by microbiological analyses of both samples taken from the periprotetic tissues and of the grafts themselves after their surgical substitution. At least one staphylococcal strain was isolated from each sample.

Of these fifteen patients 11 showed overt clinical signs of infection while 4 had only non specific clinical signs of infection but were positive at scintigraphy after injection of ^{99m}Tc -labelled leukocytes.

Sera from 10 adult, healthy subjects of both sex were used as negative controls.

Reactivity of the sera was assayed in an ELISA format, against:

- a- monospecific biofilms formed by different staphylococcal strains (both clinical isolates and reference strains) formed on small polypropylene cylinders (0.5 x 1 mm);
- b- polispecific biofilms formed by different staphylococcal strains (both clinical isolates and reference strains) formed on small polypropylene cylinders (0.5 x 1 mm);
- c- surface proteins extracted from cultures of different staphylococcal strains.

- d- the polysaccharides described in the present invention, extracted from cultures of *Staphylococcus aureus* DSM 11942 and of *Staphylococcus epidermidis* SA 1545 (see example 1 for characterization of this strain).

5 Experiments yielded the following results:

- a- both IgG and IgM antibody titers against monospecific or polispecific staphylococcal biofilms or staphylococcal surface and secretory proteins were not significantly different in either infected and non infected patients;

- 10 b- both IgG and IgM antibody titers of against the staphylococcal polysaccharide of the invention were significantly different in infected and non infected patients; they moreover allowed to differentiate between overtly symptomatic infected patients and pauci-symptomatic, scintigraphy positive patients.

15 Table 1. Range, Mean and standard deviation of both IgG and IgM titers obtained in ELISA assays performed on serum samples of 11 patients with a symptomatic vascular graft infection, 4 patients with a pauci-symptomatic vascular graft infection but positive for scintigraphy after injection of ^{99m}TC labelled leukocytes, and 10 healthy control subjects,
20 using the polysaccharide extracted from cultures of *S.aureus* DSM 11942 to sensitize microtiter wells.

Table 1

DSM 11942							
Patient Category (n.)	IgG 1/160 Range	IgG 1/160 Mean	IgG 1/160 SD*	IgM 1/160 Range	IgM 1/160 Mean	IgM 1/160 SD*	IgM 1/160
Infected Symptomatic (11)	0.262-1.06	0.66	0.25	0.32-1.032	0.66	0.27	
Non Infected (10)	0.177-0.266	0.17	0.03	0.101-0.195	0.14	0.04	
Infected pauci-symptomatic + scintigraphy (4)	0.251-0.457	0.32	0.09	0.332-0.56	0.47	0.09	

*Standard deviation

Table 2. Range, Mean and standard deviation of both IgG and IgM titers obtained in ELISA assays performed on serum samples of 11 patients with a symptomatic vascular graft infection, 4 patients with a pauci-symptomatic vascular graft infection but positive for scintigraphy after injection of ^{99m}TC labelled leukocytes, and 10 healthy control subjects, using the polysaccharide extracted from cultures of *S.epidermidis* SA 1545 to sensitize microtiter wells.

Table 2

SA 1545						
Patient Category (n.)	IgG 1/160 Range	IgG 1/160 Mean	IgG 1/160 SD*	IgM 1/160 Range	IgM 1/160 Mean	IgM 1/160 SD*
Infected Symptomatic (11)	0.173-0.971	0.47	0.21	0.31-1.071	0.56	0.2
Non Infected (10)	0.11-0.265	0.15	0.05	0.126-0.243	0.16	0.03
Infected pauci-symptomatic scintigraphy + (4)	0.202-0.445	0.29	0.11	0.287-0.483	0.37	0.06

*Standard deviation

In a second set of experiments a larger number of sera was assayed to determine both IgG and IgM titers in ELISA tests performed using only the polysaccharide of the invention to sensitize microtiter wells. All throughout these experiments the polysaccharide extracted from strain SA1545 (described in example 1) was used.

A total of 97 sera were tested in these experiments. Of these 97 sera, 19 were obtained from control patients not infected and not carrying a vascular graft, 3 were obtained from control patients not infected but carrying a vascular graft, 5 were obtained from patients carrying a vascular graft infected by Gram-negative bacteria, 13 were obtained from patients not infected but carrying a vascular graft and with a previous history of vascular graft infection caused by *Staphylococcus* spp., and 57 were obtained from patients carrying a vascular graft infected by *Staphylococcus* spp.

Both IgG and IgM titers were determined in all these sera using 1/160 and 1/320 dilutions of the sera; two sets of duplicate determinations were performed to assess intra-experiment and inter-experiment reproducibility of the assay. ELISA assays were performed as described in example 4.

Results obtained in this second set of experiments are summarized in table 3.

Table 3. Results of Enzyme Linked Immunosorbent Assays (ELISA) performed using the polysaccharide preparation of *S. epidermidis* SA1545 on 97 serum samples.

The IgM titres of each serum sample used for the statistical evaluation were mean values of four different determinations obtained in two different experiments. The IgG titres of each serum sample used for the statistical evaluation were mean values of two different determinations obtained in one experiment.

Table 3

Patient category	Mean IgM titre (1:160) (range)	Mean IgM titre (1:320) (range)	Mean IgG titre (1:160) (range)	Mean IgG titre (1:320) (range)
A1) Control patients not infected and not carrying a vascular graft (n = 19)	0.189 ± 0.037 (0.148 – 0.250)	0.136 ± 0.024 (0.116 – 0.155)	0.923 ± 0.18 (0.744 – 1.469)	0.692 ± 0.21 (0.293 – 1.198)
A2) Control patients not infected but carrying a vascular graft (n = 3)	0.153 ± 0.018 (0.134 – 0.167)	0.111 ± 0.006 (0.105 – 0.116)	1.083 ± 0.410 (0.706 – 1.585)	0.823 ± 0.356 (0.476 – 1.243)
A) Total control patients not infected (n = 7)	0.184 ± 0.037 (0.134 – 0.250)	0.133 ± 0.024 (0.105 – 0.155)	0.945 ± 0.22 (0.706 – 1.585)	0.709 ± 0.23 (0.293 – 1.243)

CONTINUED

CONTINUED TABLE 3

B) Patients carrying a vascular graft infected by Gram-negative bacteria (n = 5)	0.227 ± 0.063 (0.128 - 0.291)	0.142 ± 0.038 (0.084 - 0.174)	1.041 ± 0.265 (0.763 - 1.480)	0.700 ± 0.259 (0.420 - 1.117)
C) Patients not infected but carrying a vascular graft and with a previous history of vascular graft infection caused by <i>Staphylococcus</i> spp. (n = 13)	0.254 ± 0.076 (0.185 - 0.379)	0.169 ± 0.055 (0.107 - 0.325)	1.152 ± 0.424 (0.513 - 1.689)	0.894 ± 0.527 (0.317 - 1.652)
D) Patients carrying a vascular graft infected by <i>Staphylococcus</i> spp. (n = 57)	0.642 ± 0.516 (0.258 - >2.5)	0.377 ± 0.396 (0.127 - 2.448)	1.532 ± 0.278 (0.765 - 1.976)	1.303 ± 0.332 (0.522 - 2.013)

Results of Student T test for comparison of IgM titres measured at serum dilution = 1:160:

A(A1+A2) vs. D: $p = 1.7 \times 10^{-15}$.

B vs. D: $p = 2.0 \times 10^{-4}$.

5 C vs. D: $p = 7.2 \times 10^{-8}$.

A+B+C vs. D: $p = 5.3 \times 10^{-23}$.

A+C vs. B: $p = 0.142$.

A vs. C: $p = 2.07 \times 10^{-11}$.

10 Results of Student T test for comparison of IgG titres measured at serum dilution 1:320:

A(A1+A2) vs. D: $p = 4,5 \times 10^{-21}$.

B vs. D: $p = 7.5 \times 10^{-8}$.

C vs. D: $p = 8.0 \times 10^{-7}$.

A+B+C vs. D: $p = 3.1 \times 10^{-21}$.

15 A+C vs. B: $p = 0.265$.

A vs. C: $p = 0.024$.

Comments:

20 • The antibody titres detectable against the slime polysaccharide (SP) of *S. epidermidis* SA1545 showed significant differences in groups of different subjects, according to their clinical history.

25 • Significantly higher antibody titres against the SA1545-SP were found in patients carrying vascular grafts infected by *Staphylococcus* spp., as compared to: i) control subjects with no history of such infection, either carrying a vascular graft or not; ii) patients carrying a vascular graft infected by Gram-negative bacteria; iii) subjects carrying a vascular graft not infected, but reporting a previous history of vascular graft infection by *Staphylococcus* spp. These data overall indicate that vascular graft infection caused by *Staphylococcus*

spp. elicit a specific humoral immune response against the SP, and that this response can be detected by enzyme immunoassays using a solid phase sensitized with the SA1545 SP preparation, as described in the patent application.

- 5 • Analysis of the isotypic response anti-SA1545 SP in the above groups of subjects showed significant differences of both IgM and IgG antibody titres. The IgM titres were most closely related with the state of active infection caused by *Staphylococcus* spp.

10 The existence of a statistically significant difference between titers of infected and non infected patients allows to establish a break point value over which it is possible to define a high probability of the existence of an infective process. It is moreover possible to use this method to monitor the patient after insertion of the graft, using the antibody titer measured at the time of implantation as a specific reference value in the follow up.

15 It appears clear that the above described method has several advantages, as compared to conventional diagnostic methods in this field, since it is easy to perform, inexpensive and allows to obtain reliable results (with no need of invasive procedures) even in the early phases of infection, when all the other available methods frequently fail to give clear diagnostic
20 informations; this method moreover allows a periodic monitoring of patients for the occurrence of latent infections and could give reliable informations on therapeutic outcomes.

 The invention finally provides a kit to perform the assay, which includes the polysaccharide preparation, standard antibodies and reagents
25 for detection in adequate containers together with vehicles, excipients and additives like preservatives and stabilizers.

 Preferably the kit will contain microtiter strips pre-sensitized with the antigen together with positive and negative control sera (with their original

titres).

The following examples are intended to better clarify the invention.

Example 1

Characterization of a bacterial strain adequate for the assay.

5 A *Staphylococcus epidermidis* strain, indicated as SA1545 – yielding the numeric code 6704773 when tested for identification with the API 20 STAPH identification system, was isolated from an aorto-bifemoral graft explanted from an adult male.

10 The isolate showed an evident dimorphism of colonies when grown on Columbia Agar plates supplemented with 5% defibrinated sheep blood. It was negative for mannitol fermentation when grown on mannitol salt agar, with colonies showing a 1 mm or less of diameter after 18 h of incubation at 37°C.

15 The isolate was sensitive to the following antibiotics, as assessed using the Kirby Bauer assay: Gentamycin, Vancomycin, Ofloxacin, Erytromycin, Imipenem, Cephalotin, Amoxicillin+clavulanic acid, cefoperazone.

The biochemical pattern of the isolate is as follows:

	Fermentation	Glucose	+
20	Fermentation	Fructose	+
	Fermentation	Maltose	+
	Fermentation	Lactose	+
	Fermentation	Trehalose	-
	Fermentation	Mannitol	-
25	Fermentation	Xylitol	-
	Fermentation	Melibiose	-
	Fermentation	Raffinose	-
	Fermentation	Xylose	-

	Fermentation	Saccharose	+
	Production	Nitrates	-
	Production	Alkaline phosphatase	-
	Production	Acetoin	-
5	Production	N-acetyl-glucosaminidase	-
	Production	Arginine hydrolase	+
	Production	Urease	+

In many other cases strains showing similar characteristics and adequate to be used for the assay of the invention have been isolated.

10

Example 2

Preparation of the culture medium

The culture medium contains the following compounds per 1 liter:

	Na ₂ HPO ₄ (2H ₂ O)	10 g
	KH ₂ PO ₄	3 g
15	L-aspartic Acid	150 mg
	L-alanine	100 mg
	L-arginine	100 mg
	L-cystine	50 mg
	Glycine	100 mg
20	L-glutammic Acid	150 mg
	L-hystidine	100 mg
	L-isoleucine	150 mg
	L-lysine	100 mg
	L-leucine	150 mg
25	L-metionine	100 mg
	L-phenylalanine	100 mg
	L-proline	150 mg
	L-serine	100 mg

	L-threonine	150 mg
	L-tryptophane	100 mg
	L-tyrosine	100 mg
	L-valine	150 mg
5	Glucose	10 g
	MgSO ₄ (7H ₂ O)	500 mg
	Biotine	0,1 mg
	nicotinic Acid	2 mg
	D-pantotenic Acid	2 mg
10	Pyridoxal	4 mg
	Pyridoxamine	4 mg
	Riboflavin	2 mg
	Tiamine	2 mg
	Adenine	20 mg
15	Guanine	20 mg
	CaCl ₂ (6H ₂ O)	10 mg
	MnSO ₄	5 mg
	(NH ₄) ₂ SO ₄ FeSO ₄ (6H ₂ O)	6 mg

The formulation of the medium corresponds to the one described in
 20 Hussain, Hastings, White, J. Med. Microbiol. 34:143-147, 1991, with the
 following modifications, pertaining to the preparation: after all components
 of the medium are weighed, MgSO₄(7H₂O) is dissolved in distilled water,
 then all remaining components are added singularly, under continuous
 shaking. L-cystine prior to addition to the medium must be dissolved in 2-3
 25 drops of 5N NaOH.

Once all components are dissolved (though a small amount of
 precipitate can persist) the volume is adjusted to 1l with distilled water and
 sterilized by filtration through 0,2µm porous membranes.

Example 3

Preparation of the polysaccharide.

Strains are grown in 1000 ml of modified HHW medium for 6 days at 37°C with shaking.

5 The bacterial pellet is collected by centrifugation at 13,000 x g for 15 minutes at 4°C, suspended in 20 ml of sterile ice-cold physiological saline (NaCl 0.9%), and freezed at -20°C for 2h. The bacterial suspension is then thawed at room temperature and homogenized 10 times for 30 seconds with 30 seconds intervals.

10 The homogenate is then centrifuged at 13,000 x g for 15 minutes at 4°C; the resulting surnatant is stored at 4°C, while the resulting pellet is again suspended in 20 ml of sterile ice-cold saline and homogenized as above described. The surnatant resulting from the second homogenization step is added to the previously obtained one and desalted by dialysis against
15 1,000 volumes of distilled water at 4°C for 2 h, using diylisis membranes with a cut-off of 12 kDa. The sample is then freezed at -80°C, lyophilized, suspended in 5% (W/V) trichloroacetic acid and incubated 15 minutes at 4°C.

20 The sample is then centrifuged at 30,000 x g for 30 minutes at 4°C; 4 volumes of ice-cold absolute ethanol are then added to the surnatant, that is further incubated at 4°C for 48h. The bulk polysaccharide is then collected by centrifugation at 20,000 x g for 30 minutes at 4°C, washed with 0.5 volumes of ice-cold absolute ethanol, dehydrated under vacuum and finally suspended in 2 ml of sterile distilled water. The polysaccharide obtained as
25 above described is used to sensitize microtiter wells after adequate dilution (typically in 50 µl aliquots).

Example 4

Execution of the ELISA assay.

The wells of a microtiter plate are sensitized with 50 µl of the polysaccharide prepared according to example 3 and diluted 1/80 in sterile distilled water, adequately sealed and incubated 18-24 hours at 4°C. After incubation the wells are emptied and washed 5 times with 100 µl of 0,05% tween 20 in phosphate buffered saline (PBS).

Wells are then emptied, saturated with 200 µl of 10% soy milk in PBS, adequately sealed and incubated at 37°C for 1 hour. Wells are then emptied, washed as above described, and 50 µl aliquots of the diluted sera are added. Sera, including one positive control and one negative control, are typically diluted 1/160 and 1/320 in PBS. After addition of the sera the wells are adequately sealed and incubated at 37°C for 1 hour. Wells are then emptied, washed as above described, and 50 µl of either "Peroxidase-Conjugated Rabbit Anti-human IgG" (for example DAKO cod. P0214) (diluted 1/15,000 in 10% soy milk in PBS) or "Peroxidase Conjugated Rabbit anti-Human IgM" (for example DAKO cod. P0215) (diluted 1/1,500 in 10% soy milk in PBS) are added. Wells are then adequately sealed, incubated at 37°C for 1 hour, emptied and washed as above described.

Following last washing 50 µl of the chromogenic substrate for peroxidase (for example BM Blue POD Substrate Boehringer Mannheim, cod 1484281) are added to each well.

The wells are then incubated 10 minutes at 37°C and the reaction is then stopped by adding 50 µl of 0.5N H₂SO₄. The OD_{450nm} of each well is then evaluated using a microtiter plate reader using one untreated well containing 100 µl of 0.5N H₂SO₄ as the blank.

As a general rule OD_{450nm} for positive control should not be over the value 1.5.

Should this happen the assay must be repeated, reducing the time of incubation of the peroxidase substrate.

BRACCO S.p.A.
Via Folli, 50
I-20134 Milano

EMPFANGSBESTÄTIGUNG BEI ERSTHINTERLEGUNG,
ausgestellt gemäß Regel 7.1 von der unten angegebenen
INTERNATIONALEN HINTERLEGUNGSSTELLE

I. KENNZEICHNUNG DES MIKROORGANISMUS

Vom HINTERLEGER zugewiesenes Bezugszeichen:

6538P

Von der INTERNATIONALEN HINTERLEGUNGSSTELLE
zugewiesene EINGANGSNUMMER:

DSM 11942

II. WISSENSCHAFTLICHE BESCHREIBUNG UND/ODER VORGESCHLAGENE TAXONOMISCHE BEZEICHNUNG

Mit dem unter I. bezeichneten Mikroorganismus wurde

- () eine wissenschaftliche Beschreibung
(X) eine vorgeschlagene taxonomische Bezeichnung

eingereicht.
(Zureichendes ankreuzen).

III. EINGANG UND ANNAHME

Diese internationale Hinterlegungsstelle nimmt den unter I bezeichneten Mikroorganismus an, der bei ihr am 1998-01-20 (Datum der Erstinbringung)¹ eingegangen ist.

IV. EINGANG DES ANTRAGS AUF UMWANDLUNG

Der unter I bezeichnete Mikroorganismus ist bei dieser internationalen Hinterlegungsstelle am eingegangen (Datum der Erstinbringung) und ein Antrag auf Umwandlung dieser Erstinbringung in eine Hinterlegung gemäß Budapest-Vertrag ist am eingegangen (Datum des Eingangs des Antrags auf Umwandlung).

V. INTERNATIONALE HINTERLEGUNGSSTELLE

Name: DSMZ-DEUTSCHE SAMMLUNG VON
MIKROORGANISMEN UND ZELLKULTUREN GmbH

Anschrift: Mascheroder Weg 1b
D-38124 Braunschweig

Unterschrift(en) der zur Vertretung der internationalen Hinterlegungsstelle
befugten Person(en) oder des (der) von ihr ermächtigten Bediensteten:



Datum: 1998-01-22

¹ Falls Regel 6.4 Buchstabe d zutrifft, ist dies der Zeitpunkt, zu dem der Schutz einer internationalen Hinterlegungsstelle erworben worden ist.

CLAIMS

1. A method for the determination of prosthetic infections in which at least one *Staphylococcus* strain is involved, which comprises detecting from blood samples or other biological fluid samples antibodies reacting with a polysaccharide produced by a virulent staphylococcal strain.
2. A method according to claim 1, in which the antibodies are IgG and IgM.
3. A method according to claims 1-2, in which the virulent staphylococcal strain is a strain of coagulase negative or positive species.
4. A method according to claim 3, in which said species is *Staphylococcus epidermidis* or *Staphylococcus aureus*.
5. A method according to claim 3, in which the virulent staphylococcal strain is DSMZ No. 11942.
6. A method according to claims 1-5, in which the polysaccharide is obtained by the following steps:
 - a) culturing the staphylococcal strains in modified HHW medium for a period of 4-6 days;
 - b) homogenizing the bacterial cells in a physiological buffer;
 - c) centrifugating at 13,000 x g for 15 minutes and separating the surnatants;
 - d) desalting by dialysis the surnatant using membranes with a cut-off of 12 kDa;
 - e) freezing and lyophilizing the solution obtained in (d);
 - f) suspending the lyophilized material in a deproteinizing solution;
 - g) centrifugating at 30,000xg the solution obtained in (f) and separating the surnatant with addition of ethanol;
 - h) centrifugating the surnatant of step (g) at 20,000xg to obtain the

polysaccharide;

- i) washing the precipitated polysaccharide with absolute ethanol,
dehydrating in vacuo and suspending it in sterile H₂O.

5 7. A method according to claims 1-6, which is in form of ELISA, gel
immuno-precipitation, immuno-diffusion, contro-immunoelectrophoresis,
radioimmunologic assay, complement fixation.

8. A process for preparing a polysaccharide from *Staphylococcus* cultures
which comprises:

- 10 a) culturing the staphylococcal strains in modified HHW medium for a
period of 4-6 days;
- b) homogenizing the bacterial cells in a physiological buffer;
- c) centrifugating at 13,000 x g for 15 minutes and separating the
surnatants;
- 15 d) desalting by dialysis the surnatant using membranes with a cut-off of
12 kDa;
- e) freezing and lyophilizing the solution obtained in (d);
- f) suspending the lyophilized material in a deproteinizing solution;
- g) centrifugating at 30,000xg the solution obtained in (f) and separating
the surnatant with addition of ethanol;
- 20 h) centrifugating the surnatant of step (g) at 20,000xg to obtain the
polysaccharide;
- i) washing the precipitated polysaccharide with absolute ethanol,
dehydrating in vacuo and suspending it in sterile H₂O.

9. A polysaccharide obtainable by the process of claim 6.

25 10. A kit for use in a method according to claims 1-5, containing the
polysaccharide, the antibodies and the detection reagents in suitable
containers in combination with vehicles, excipients, additives, preservatives
or stabilizers.

11. A kit according to claim 8, containing microtiter strips pre-sensitized with the antigen together with positive and negative control sera.
12. Use of a polysaccharide produced by virulent staphylococcal strains in an immunochemical assay for the determination of prosthetic infections.
- 5 13. Use according to claim 12, wherein the polysaccharide is that of claim 9.
14. Staphylococcal strain deposited at DSMZ under deposit No. 11942.

Case No. _____

Nixon & Vanderhye P.C. (12/97)

RULE 63 (37 C.F.R. 1.63)
DECLARATION AND POWER OF ATTORNEY
FOR PATENT APPLICATION
IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

As a below named inventor, I hereby declare that my residence, post office address and citizenship are as stated below next to my name, and I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

Method for the determination of prosthetic infections

the specification of which (check applicable box(es)):

- ☐ is attached hereto
☐ was filed on _____ as U.S. Application Serial No. _____

X was filed as PCT International application No. PCT/EP99/00618 on 01-02-1999

and (if applicable to U.S. or PCT application) was amended on _____

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above. I acknowledge the duty to disclose information which is material to the patentability of this application in accordance with 37 C.F.R. 1.56. I hereby claim foreign priority benefits under 35 U.S.C. 119/365 of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed or, if no priority is claimed, before the filing date of this application:

Priority Foreign Application(s):

Application Number	Country	Day/Month/Year Filed
MI98A000197	Italy	03-02-1998

I hereby claim the benefit under 35 U.S.C. §119(e) of any United States provisional application(s) listed below.

Application Number	Date/Month/Year Filed
--------------------	-----------------------

I hereby claim the benefit under 35 U.S.C. 120/365 of all prior United States and PCT international applications listed above or below and, insofar as the subject matter of each of the claims of this application is not disclosed in such prior applications in the manner provided by the first paragraph of 35 U.S.C. 112, I acknowledge the duty to disclose material information as defined in 37 C.F.R. 1.56 which occurred between the filing date of the prior applications and the national or PCT international filing date of this application:

Prior U.S./PCT Application(s):

Application Serial No.	Day/Month/Year Filed
------------------------	----------------------

Status: patented
pending, abandoned

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon. And I hereby appoint **NIXON & VANDERHYE P.C., 1100 North Glebe Rd., 8th Floor, Arlington, VA 22201-4714, telephone number (703) 816-4000 (to whom all communications are to be directed)**, and the following attorneys thereof (of the same address) individually and collectively my attorneys to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith and with the resulting patent: Arthur R. Crawford, 25327; Larry S. Nixon, 25640; Robert A. Vanderhye, 27076; James T. Hosmer, 30184; Robert W. Faris, 31352; Richard G. Besha, 22770; Mark E. Nusbaum, 32348; Michael J. Keenan, 32106; Bryan H. Davidson, 30251; Stanley C. Spooner, 27393; Leonard C. Mitchard, 29009; Duane M. Byers, 33363; Jeffry H. Nelson, 30481; John R. Lastova, 33149; H. Warren Burnam, Jr., 29366; Thomas E. Byrne, 32205; Mary J. Wilson, 32955; J. Scott Davidson, 33489; Alan M. Kagen, 36178; William J. Griffin, 31260; Robert A. Molan, 29834; B. J. Sadoff, 36663; James D. Berquist, 34776; Updeep S. Gill, 37334.

1. Inventor's Signature: [Signature] Date: 09.09.2000
Inventor: Maria Cristina THALLER Italian
(first) MI (last) (citizenship)

Residence: (city) Milano (state/country) Italy ITX
Post Office Address: Via E. Folli, 50 - Milano, Italy
(Zip Code) 20134

2. Inventor's Signature: [Signature] Date: 09.09.2000
Inventor: Gianmaria ROSSOLINI Italian
(first) MI (last) (citizenship)

Residence: (city) Milano (state/country) Italy ITX
Post Office Address: Via E. Folli, 50 - Milano, Italy
(Zip Code) 20134

3. Inventor's Signature: [Signature] Date: 09.09.2000
Inventor: Laura SELAN Italian
(first) MI (last) (citizenship)

Residence: (city) Milano (state/country) Italy ITX
Post Office Address: Via E. Folli, 50 - Milano, Italy
(Zip Code) 20134

FOR ADDITIONAL INVENTORS, check box X and attach sheet with same information and signature and date for each.

RULE 63 (37 C.F.R. 1.63)
DECLARATION AND POWER OF ATTORNEY
FOR PATENT APPLICATION
IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

4.	Inventor's Signature: <u><i>C. Passariello</i></u> Date: <u>09.09.2000</u>				
H-00	Inventor: <table border="0" style="width: 100%;"><tr><td style="width: 35%;"><u>Claudio</u> (first)</td><td style="width: 10%; text-align: center;">MI</td><td style="width: 35%;"><u>PASSARIELLO</u> (last)</td><td style="width: 20%;"><u>Italian</u> (citizenship)</td></tr></table>	<u>Claudio</u> (first)	MI	<u>PASSARIELLO</u> (last)	<u>Italian</u> (citizenship)
<u>Claudio</u> (first)	MI	<u>PASSARIELLO</u> (last)	<u>Italian</u> (citizenship)		
	Residence: (city) <u>Milano</u> (state/country) <u>Italy</u> <u>ITX</u>				
	Post Office Address: <u>Via E. Folli, 50 - Milano, Italy</u>				
	(Zip Code) <u>20134</u>				
5.	Inventor's Signature: _____ Date: _____				
	Inventor: _____ (first) MI (last) (citizenship)				
	Residence: (city) _____ (state/country) _____				
	Post Office Address: _____				
	(Zip Code) _____				
6.	Inventor's Signature: _____ Date: _____				
	Inventor: _____ (first) MI (last) (citizenship)				
	Residence: (city) _____ (state/country) _____				
	Post Office Address: _____				
	(Zip Code) _____				
7.	Inventor's Signature: _____ Date: _____				
	Inventor: _____ (first) MI (last) (citizenship)				
	Residence: (city) _____ (state/country) _____				
	Post Office Address: _____				
	(Zip Code) _____				
8.	Inventor's Signature: _____ Date: _____				
	Inventor: _____ (first) MI (last) (citizenship)				
	Residence: (city) _____ (state/country) _____				
	Post Office Address: _____				
	(Zip Code) _____				
9.	Inventor's Signature: _____ Date: _____				
	Inventor: _____ (first) MI (last) (citizenship)				
	Residence: (city) _____ (state/country) _____				
	Post Office Address: _____				
	(Zip Code) _____				
10.	Inventor's Signature: _____ Date: _____				
	Inventor: _____ (first) MI (last) (citizenship)				
	Residence: (city) _____ (state/country) _____				
	Post Office Address: _____				
	(Zip Code) _____				
11.	Inventor's Signature: _____ Date: _____				
	Inventor: _____ (first) MI (last) (citizenship)				
	Residence: (city) _____ (state/country) _____				
	Post Office Address: _____				
	(Zip Code) _____				
12.	Inventor's Signature: _____ Date: _____				
	Inventor: _____ (first) MI (last) (citizenship)				
	Residence: (city) _____ (state/country) _____				
	Post Office Address: _____				
	(Zip Code) _____				